PHOTOSYNTHETIC PIGMENTS OF SYMBIOTIC DINOFLAGELLATES (ZOOXANTHELLAE) FROM CORALS AND CLAMS[1]

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The brown symbiotic algae, or zooxanthellae, that live in great numbers in the tissues of some marine invertebrates, are considered on morphological evidence to be closely related to the dinoflagellates (Klebs, 1884; Kawaguti, 1944; Pringsheim, 1955). In the animal tissues the cells are in a non-motile vegetative state, without flagella or well-defined girdle, but upon isolation from the host tissue some zooxanthellae develop into typical gymnodinioid swarmers (Zahl and McLaughlin, 1957; McLaughlin and Zahl, 1959). Freudenthal (1962) has studied transformations in great detail in zooxanthellae from the jellyfish *Cassiopea* sp., and he has created a new genus, *Symbiodinium microadriaticum,* within the Dinophyceae, to accommodate this zooxanthella form. Droop (1963), however, has pointed out that "it is by no means certain that all zooxanthellae are dinoflagellates," and McLaughlin and Zahl (1966) warned that "caution must be exercised in the matter of speciation."

Knowledge of the photosynthetic pigment composition of algae can, when taken together with morphological evidence, provide a firm basis for the recognition of group affinities. This biochemical approach becomes increasingly powerful as gaps in our knowledge of the pigment composition of rare as well as known algal groups are filled. Studies of zooxanthellae pigments have, however, been relatively few. Present knowledge of the pigment composition of zooxanthellae rests largely upon studies by Strain, Manning, and Hardin (1944) of the pigment composition of the free-living dinoflagellate *Peridium cinctum* and the endosymbiont of the sea-anemone *Bunodactis xanthogrammica* (now *Anthopleura xanthogrammica; cf.* Ricketts and Calvin, 1962). Using chromatography on columns of powdered sucrose they found that the pigment composition of the zooxanthellae exactly paralleled that of *Peridinium* in possessing chlorophylls *a* and *c*, β-carotene, and the xanthophylls peridinin and neo-peridinin, diadinoxanthin and dinoxanthin. It was concluded from the pigment evidence that the *Anthopleura* zooxanthellae and the dinoflagellate *Peridinium* "may belong to the same or related plant groups." However, identity of the former as cryptomonads from morphological evidence could not be excluded at the time. Subsequent biochemical analyses of cryptomonads by Haxo and Fork (1959) and Mallams *et al.* (1967) showed that these algae possess a unique com-

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plement of biliproteins and carotenoids, and thus any relationship between zooxanthellae and cryptomonads seems now to be completely excluded.

Earlier, Heilbron, Jackson and Jones (1935) crystallized a red-orange pigment from the sea-anemone *Anemonia sulcata* which they called sulcatoxanthin. Strain *et al.* (1944) recognized that sulcatoxanthin was probably identical with peridinin, especially since this anemone is known to contain large numbers of zooxanthellae (Stephenson, 1935).

During the present Expedition [1] a unique opportunity was provided to examine the photosynthetic pigments of the exotic and little studied tridacnid clam zooxanthellae, and an assortment of coral endosymbionts. The pigments were screened by a simple two-dimensional paper chromatographic method (Jeffrey, 1961), which gave reliable separations of the chlorophylls and carotenoids, and which could be used, together with spectral analysis, as a simple aid to the identification of the pigments present. The pigments of the dinoflagellates *Amphidinium* and *Gymnodinium* spp. were used as standard reference material. The zooxanthellae from

TABLE I

Corals used in zooxanthellae survey

CLASS: Anthozoa; *SUB-CLASS*: Zooantharia; *ORDER*: Scleractinia

i) *Acropora* sp.
ii) *Seriatopora* sp.
iii) *Favia* sp.
iv) *Fungia* (two species)
v) *Pocillopora* sp.

SUB-CLASS: Alcyonaria; *ORDER*: Coenothecalia
vi) *Heliopora* sp.

SUB-CLASS: Alcyonaria; *ORDER*: Alcyonacea
vii) *Xenia* sp. (two species)

CLASS: Hydrozoa; *ORDER*: Milleporina
viii) *Millepora* sp.

five species of tridacnid clams, eight zooantharian and alcyonarian corals and one hydrozoan coral were found to have an identical pigment pattern to that of the dinoflagellate *Amphidinium*. The organisms contained chlorophylls *a* and *c*, β-carotene, peridinin and neo-peridinin, dinoxanthin and diadinoxanthin, and three other unidentified xanthophylls present in very small quantities.

MATERIALS AND METHODS

MATERIALS

Clams and corals were collected from the Great Barrier Reef just outside Princess Charlotte Bay, North Queensland. Species of tridacnid clams studied were *Tridacna crocea, Tridacna gigas, Tridacna squamosa, Tridacna deresa, Tridacna maxima,* and *Hippopus hippopus*. Identifications were made according to the descriptions provided by Rosewater (1965). Nine corals from both major classes and sub-classes were studied (Table I).

METHODS

A. *Preparation of zooxanthellae*

Zooxanthellae were isolated from clam mantle tissue and corals according to methods worked out by Muscatine (1967).

1. *Clams.* Mantle tissue containing zooxanthellae was excised, and freed as much as possible from supporting muscle tissue. The mantle was rinsed several times in filtered sea water, and cut into small pieces. The tissue was homogenized in sea water in a Waring Blendor for one minute, and the resulting suspension was filtered through six layers of cheesecloth, to free the cells from tissue debris. The dark brown suspension of algal cells was washed three times by centrifuging in filtered sea water at about 2500 *g* for five minutes. The cells were finally resuspended in sea water to a homogeneous suspension. Packed cells could be stored frozen without pigment deterioration for several weeks.

2. *Corals.* Zooxanthellae from hard corals were released by crushing the tissue into small pieces in aluminium foil with hammer or pliers, and rinsing continuously in sea water to wash out the cells. The suspension was filtered through cheesecloth, and the cells washed as before by centrifuging 2–3 times in filtered sea water.

Zooxanthellae from the tentacles of soft corals (*Xenia* spp.), and from the tentacles of *Fungia,* were collected by excising the tentacles and grinding them gently in sea water in a glass Potter-Elvehjem homogeniser. The zooxanthellae, which were released intact by this treatment, were filtered through cheesecloth, and washed by centrifugation as above. In every case, beautiful, clean suspensions of undamaged zooxanthellae were obtained.

B. *Preparation of pigment extracts*

Zooxanthellae were extremely difficult to extract in either acetone or methanol at room temperature. Pigments were readily released, however, if the packed cells were first suspended in a little distilled water, and frozen for 4–8 hours before methanol extraction. This procedure apparently weakened the tough cell wall, and allowed subsequent extraction to proceed readily, without alteration of the pigments. The cells were extracted about 2–3 times in methanol, until the residue was colorless. The methanol extract was clarified by centrifugation, mixed with an equal volume of diethyl ether, and shaken once or twice with a volume of 10% NaCl solution 5–10 times that of the methanol-ether extract. This saline washing caused the pigments to migrate to the ether layer, and methanol and methanol-soluble impurities were washed out in the aqueous phase. The ether layer was then concentrated for chromatography by evaporation under a stream of nitrogen. The ether extracts could be stored in the deep freeze for several days without deterioration of the pigments.

Some of the hard corals were extremely difficult to crush for the collection of zooxanthellae. Small pieces of these corals were extracted "whole" with methanol for several hours at room temperature, in the presence of a little $MgCO_3$, to prevent acidification. Extraction was continued until the coral skeleton was colorless. Pigments were not damaged by this treatment, as was shown by subsequent chromatography.

The dinoflagellates, *Amphidinium* and *Gymnodinium*, were used as standard sources of dinoflagellate pigments. *Gymnodinium* was used mainly as a source of standard dinoxanthin, since in *Amphidinium* the amount of this pigment was extremely small. Cultures were grown in Medium f (Guillard; see Grant, 1967) for about two weeks at 5000 lux. The cells were harvested by continuous centrifugation, and pigments were readily extracted from the packed cells with small volumes of 90% acetone. After 2–3 extractions the residue was colorless. Pigments were transferred from acetone to ether as above, and used directly for chromatography. Samples of *Amphidinium* cells were freeze-dried, for dispatch from Cronulla to Princess Charlotte Bay. The dry powder could be stored in the dark at room temperature for several weeks, without deterioration of the pigments.

C. *Paper chromatography*

Pigment extracts were chromatographed on 22-cm. squares of Whatman No. 3 (or No. 3 Mm paper in two dimensions, according to the method of Jeffrey (1961). Solvents used were AR Grade, and were not further purified. Solvent systems used were 4% *n*-propanol in light petroleum (60–80° C.) for the first dimension, and 30% $CHCl_3$ in light petroleum for the second dimension. Absorption spectra of pigment fractions were obtained by running a number of chromatograms, and eluting the pigment spots. Absorption spectra were taken with a Beckman DB spectrophotometer (for work done on the "Alpha Helix"), a Unicam SP 700 (for work done subsequently at Cronulla), and a Carey Model 14 (for work done subsequently at La Jolla). The latter instruments were calibrated with Hg lines and were considered accurate to 1 nm.

D. *Quantitative determination of pigments*

1. *Spectrophotometric.* Routine quantitative determinations of chlorophylls *a* and *c* were carried out on pigment extracts, either in methanol, 90% acetone, or in diethyl ether. Extinctions were measured at the red maxima of the two chlorophylls, and the concentration calculated from the provisional equations of Humphrey and Jeffrey (in preparation).

In ether:

$$\begin{cases} \text{chl } a = 10.5\ E_{662} - 1.0\ E_{628} \\ \text{chl } c = -8.1\ E_{662} + 64.3\ E_{628} \end{cases}$$

In 90% acetone:

$$\begin{cases} \text{chl } a = 13.31 \times E_{663} - 0.27 \times E_{630} \\ \text{chl } c = -8.37 \times E_{663} + 51.72 \times E_{630} \end{cases}$$

In methanol:

$$\begin{cases} \text{chl } a = 13.8\ E_{668} - 1.3\ E_{635} \\ \text{chl } c = -14.1\ E_{668} + 67.3\ E_{635} \end{cases}$$

where chl = concentration of chlorophyll in ug./ml. and E = extinction in liters/gm. cm. in a 1-cm. cell.

2. *Chromatographic.* The percentage composition of each carotenoid fraction was determined by running several chromatograms simultaneously, and eluting the spots immediately in the appropriate solvent. Solutions were made up to a

measured volume, centrifuged to remove any paper fibers, and the extinctions read without delay. In this, and in all the chromatographic work, operations were carried out in dim light, or in complete darkness. Photo-decomposition of pigments was thus kept at a minimum.

Concentrations of pigments were calculated using the following extinction coefficients:

Chlorophyll *a* in acetone	90 l./gm.cm.	Vernon (1960)
Chlorophyll *c* in methanol	15.2 l./gm.cm.	Jeffrey (1963)
Carotene in ether	250.5 l./gm.cm.	Goodwin (1955)
Peridinin in ethanol	132.5 l./gm.cm.	present work

The concentrations of all other carotenoids, whose extinctions were unknown, were calculated using the extinction coefficient of carotene.

E. *Thin layer chromatography*

Since paper chromatography does not resolve carotene isomers, thin layers of $Al_2O_3 + MgO$ (3:1 w/w) were used to characterize the carotene fractions. Using as solvent 4% ethyl acetate in hexane (Chapman, 1966), α- and β-carotenes were separated with R_f values of 0.67 and 0.41. α-Carotene for reference was obtained from the cryptomonad *Chloromonas* sp., and β-carotene from the green flagellate, *Dunaliella tertiolecta*.

The pink-orange xanthophyll (fraction 9), which remained at the origin in the paper chromatographic system was separated from chlorophyll *c* by chromatography of the "origin material" on thin layers of polyethylene, using 90% acetone as solvent. The pink xanthophyll ran just behind the solvent front ($R_f = 0.95$) with chlorophyll *c* at R_f values of 0.3 and 0.4. The xanthophyll was eluted with ethanol, for spectral analysis.

A thin layer chromatography system, which completely duplicated the paper chromatography method, and which gave even better resolution of all the dinoflagellate pigments, was subsequently developed after returning from the Expedition. This method (to be described in detail in a separate communication) uses plates of specially prepared sucrose, with 0.6% *n*-propanol in petroleum ether (60–80° C.) and 12% chloroform in petroleum ether, as the two-dimensional solvent system. Extracts of *Amphidinium*, and extracts of zooxanthellae from the clam *Tridacna crocea* and the coral *Pocillopora* were chromatographed using this system.

F. *Crystallization of peridinin*

Approximately 6 mg. crystalline peridinin were isolated from *Tridacna gigas* as follows. The mantle tissue was excised from a healthy giant clam, freed substantially of colorless animal tissue and immediately deep frozen. The frozen zooxanthellae-laden mantle tissue weighing some two Kg. was chopped into small pieces and exhaustively extracted in the cold with 95% ethanol. The total pigment was transferred to benzene with the addition of saturated NaCl solution, and the washed and dried pigment solution evaporated to dryness *in vacuo* at 30° C. The pigment was dissolved in 80% aqueous methanol, and the

initial separation of the peridinin from other pigments and colorless contaminants was made by passage of the methanolic solution through powdered polyethylene. The peridinin fraction was recovered as the leading zone. Crude peridinin from several such columns was transferred to benzene and then chromatographed on columns of powdered $CaCO_3$, developed with 1–2% acetone in benzene, according to the method of Pinckard *et al.* (1953). Prior to crystallization, the almost pure peridinin was rechromatographed on thin layer plates of silica gel G employing 30% acetone in hexane as developing solvent.

Crystallization of peridinin was carried out according to the procedures of Pinckard *et al.* (1953), once from benzene-hexane and once from ether-hexane mixtures. The homogeneous microcrystalline product was scarlet to warm reddish brown in color, disc-shaped and free of colorless contaminants. After drying *in vacuo* at 65.5° C. the product weighed a little over 6 mg.

The crystals were readily soluble in methanol, ethanol, acetone, ether, and benezene, and virutally insoluble in hexane. Methanolic solutions were found

TABLE II

Extinction coefficients of peridinin isolated from Tridacna gigas

Solvent		Maxima (nm)	Extinction coefficient $E_{1cm.}^{1\%}$
Acetone	(single broad maximum)	466	1340
90% Acetone	(single broad maximum)	469	1330
Methanol (absolute)	(single broad maximum)	469	1360
Ethanol (absolute)	(single broad maximum)	472	1325
Pyridine	(single broad maximum)	475	1180
Diethyl ether		454,* 475	1450
Benzene		467,* 494	1290
Hexane-ether (9:1)		454,* 483	1470
Chloroform		470,* 490	1290
Hexane		454, 484	—

* Extinctions taken at these maxima.

to be strongly hypophasic. The phase distribution in the system 65% MeOH/hexane was 96:4.

The melting point of the crystals was determined in capillaries sealed under N_2 employing a Thomas Hoover capillary melting point apparatus. Averaged values for two determinations were: softened at 130.7° C. (cor.) and complete melt at 134.9° C. (cor.). These values agree well with the total melt point of 130° C. reported by Heilbron *et al.* (1935) for sulcatoxanthin (*i.e.*, peridinin) from *Anemonia sulcata.*

For estimation of the extinction coefficient 1.040 mg. peridinin were dissolved in 200 ml. acetone and spectral absorption determined in a 1-cm. path length cuvette in the Cary 14 Spectrophotometer. From this measurement the $E_{1\,cm.}^{1\%}$ at 466 mμ, the wave-length of maximum absorption in acetone, was calculated to be 1340. The ash content of the sample is not known and the extinction coefficient is considered to be minimal. This value is substantially higher than the

extinction coefficient of 840 (acetone) reported by Parsons and Strickland (1963) for peridinin from *Amphidinium carterei*. Relative extinctions of peridinin in other solvents were determined by evaporating aliquot samples of the above acetone solution to dryness in a rotary evaporator *in vacuo* at room temperature and dissolving in the appropriate solvent. Extinction values for peridinin in different solvents, and absorption maxima, are given in Table II.

By way of further characterization of peridinin from *Tridacna gigas*, iodine-catalyzed trans-cis isomerizations similar to those performed by Pinckard *et al.*

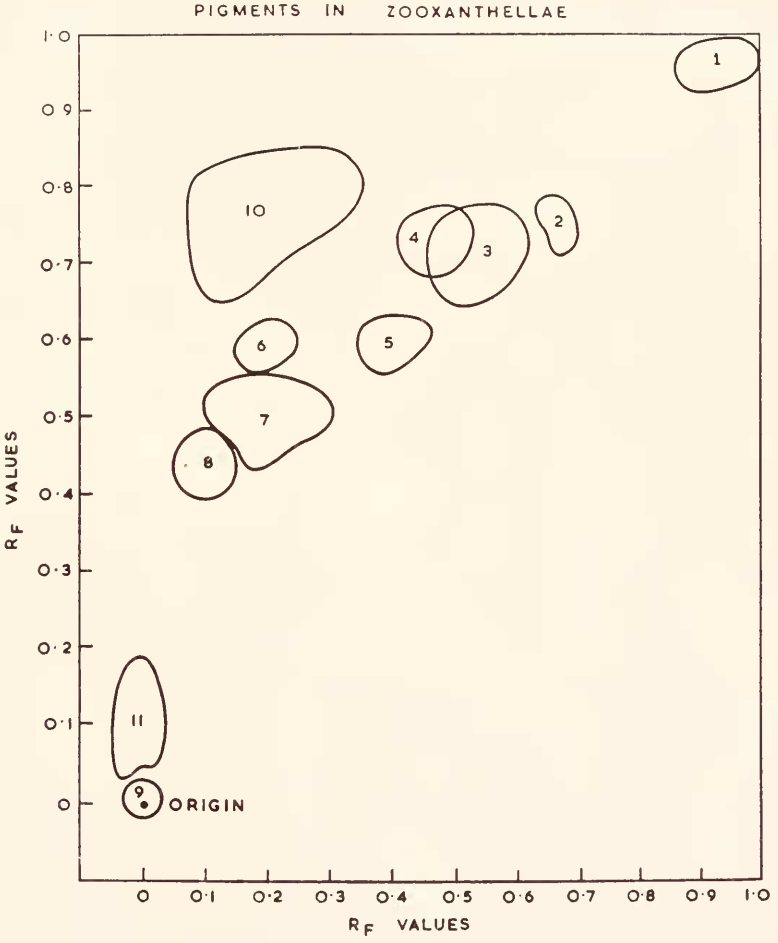

FIGURE 1. Two-dimensional paper chromatogram of pigments in zooxanthellae from *Tridacna crocea*. Chromatographic solvent systems: 1st dimension, 4% *n*-propanol in petroleum ether (60–80°); 2nd dimension, 30% chloroform in petroleum ether. 1, β-carotene (orange). 2, Unknown (pale orange). 3, Diadinoxanthin (yellow). 4, Dinoxanthin (yellow). 5, Unknown (yellow). 6, Neo-dinoxanthin (yellow). 7, Peridinin (brick red). 8, Neo-peridinin (brick red). 9, Unknown (pink orange). 10, Chlorophyll *a* (blue green). 11, Chlorophyll *c* (light green).

(1953) for crystalline peridinin isolated from *Prorocentrum micans* were carried out. In all respects examined the properties of the two preparations were identical, *e.g.*, spectra before and after iodine catalysis of benzene solutions; number, adsorption position and spectral properties of isomers separable on $CaCO_3$ columns; and percentage composition of an equilibrium mixture of stereo-isomers (57% all-trans and 43% combined cis-isomers).

For comparison with one of the original sources used by Strain *et al.* (1944), peridinin was isolated from zooxanthallae from the sea anemone *Anthopleura xanthogrammica*. (Live sea anemones were collected intertidally at La Jolla.) Peridinins from the sea anemone and *Tridacna gigas* were found to be chromatographically homogeneous in the thin layer system, silica gel G-30% acetone in hexane.

Results

Figure 1 shows a typical two-dimensional paper chromatogram of the pigments of zooxanthellae from *Tridacna crocea*. Identical chromatograms were

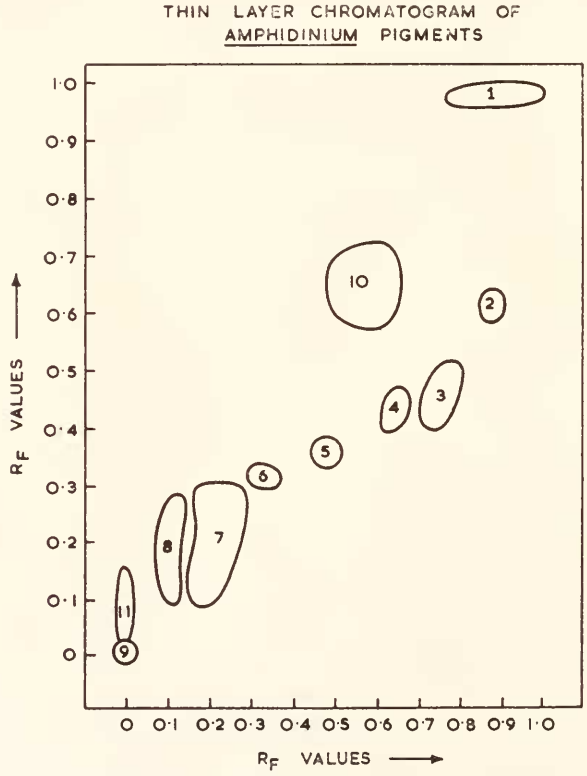

Figure 2. Sucrose thin layer chromatogram of pigments in the dinoflagellate *Amphidinium* sp. Chromatographic solvent systems; 1st dimension, 0.6% *n*-propanol in petroleum ether (60–80°); 2nd dimension, 12% chloroform in petroleum ether. Pigment fractions as in Figure 1.

also obtained from the other four clam zooxanthellae, the nine coral zooxanthellae, and the dinoflagellate *Amphidinium*. In every case chlorophyll *a* was accompanied by chlorophyll *c*, and the major carotenoids were always β-carotene, peridinin, neo-peridinin, dinoxanthin, and diadinoxanthin. Average R_f values for dinoflagellate pigments in the two solvent systems are given in Table III. The carotene fraction was shown to consist only of the β-isomer by chromatography of the carotene fraction from zooxanthellae and *Amphidinium* on thin layers of alumina and magnesium oxide.

Figure 2 shows a typical chromatogram of dinoflagellate pigments separated on specially prepared thin layers of powdered sucrose. Again, identical patterns of pigments were obtained with *Amphidinium* and zooxanthellae extracts from the clam *Tridacna crocea* and the coral *Pocillopora*. Since the thin layer method will be described in detail in a separate communication, only results obtained with the paper method, which was used on the Expedition, will be presented here.

TABLE III

R_f values of pigment fractions from zooxanthellae and Amphidinium using paper chromatography

Fraction	Pigment	Color	R_f Values	
			4% *n*-propanol in pet. ether	30% $CHCl_3$ in pet. ether
1	Carotene	Orange	0.96	0.93
2	Unknown	Pale orange	0.75	0.67
3	Diadinoxanthin	Yellow	0.71	0.54
4	Dinoxanthin	Yellow	0.73	0.48
5	Unknown	Pale yellow	0.59	0.40
6	Neo-dinoxanthin	Pale yellow	0.59	0.19
7	Peridinin	Brick red	0.49	0.19
8	Neo-peridinin	Brick red	0.43	0.10
9	Unknown	Pink-orange	0	0
10	Chlorophyll *a*	Blue-green	0.76	0.21
11	Chlorophyll *c*	Light green	0.10	0

Absorption spectra of all pigment zones were analyzed in detail in zooxanthellae from the two clams *Tridacna crocea* and *Hippopus hippopus*, the coral *Pocillopora* and the dinoflagellate *Amphidinium*. The absorption maxima are listed in Table IV, and are compared with absorption maxima of pigments from the dinoflagellate *Peridinium cinctum* given by Strain, Manning and Hardin (1944).

Absorption properties of peridinin from *Tridacna gigas* and *Amphidinium* were checked on more accurate spectrophotometers after the Expedition. The values for peridinin in ethanol obtained on the Expedition with the Beckman DB spectrophotometer were from 473–475 nm (Table IV). Using the same paper chromatography method, the maximum of peridinin in ethanol from *Amphidinium* was 474 nm (Unicam SP700), and from *Tridacna gigas* was 473 nm (Carey Model 14). The absorption maximum of *Tridacna gigas* peridinin prepared as an all-trans sample and then crystallized, was 473 nm; prepared as above but rechromatographed on $CaCO_3$ to obtain the fresh all-trans fraction was 472 nm.

TABLE IV

Absorption spectra of pigments from zooxanthellae of corals, clams, and dinoflagellates

Fraction	Organism	Absorption maxima (nm)	Solvent	Identification
Fraction 1 (orange)	*Tridacna crocea*	426, 448, 475	diethyl ether	*β-Carotene*
	Hippopus hippopus	428, 449, 475	diethyl ether	
	Pocillopora sp.	428, 448, 475	diethyl ether	
	Amphidinium sp	429, 450, 475	diethyl ether	
		429, 450, 478	hexane	
	*Peridinium cinctum**	not given		
Fraction 2 (pale orange)	*Tridacna crocea*	429, 450, 476	ethanol	*Unknown*
	Hippopus hippopus	429, 450, 478	ethanol	
	Pocillopora sp.	427, 450, 477	ethanol	
	Amphidinium sp.	429, 452, 477	ethanol	
	*Peridinium cinctum**	not present		
Fraction 3 (yellow)	*Tridacna crocea*	425, 447, 477	ethanol	*Diadinoxanthin*
	Hippopus hippopus	426, 448, 477	ethanol	
	Pocillopora sp.	425, 447, 476.5	ethanol	
	Amphidinium sp.	425, 448, 478	ethanol	
	*Peridinium cinctum**	448, 478	ethanol	
Fraction 4 (yellow)	*Tridacna crocea*	418, 442, 470	ethanol	*Dinoxanthin*
	Hippopus hippopus	418, 441, 468	ethanol	
	Pocillopora sp.	418, 441, 469	ethanol	
	Amphidinium sp.	418, 443, 472	ethanol	
	*Peridinium cinctum**	441.5, 471	ethanol	
Fraction 5 (pale yellow)	*Tridacna crocea*	408, 427, 455	ethanol	*Unknown*
	Hippopus hippopus	410, 428, 455	ethanol	
	Pocillopora sp.	408, 427, 455	ethanol	
	Amphidinium sp.	408, 427, 455	ethanol	
	*Peridinium cinctum**	not present		
Fraction 6 (pale yellow)	*Tridacna crocea*	425, 441, 467	ethanol	*Neo-dinoxanthin*
	Hippopus hippopus	425, 440, 466	ethanol	
	Pocillopora sp.	420, 440, 465	ethanol	
	Amphidinium sp.	422, 440, 467	ethanol	
	*Peridinium cinctum**	438, 466	ethanol	
Fraction 7 (brick red)	*Tridacna crocea*	475	ethanol	*Peridinin*
	Hippopus hippopus	473	ethanol	
	Pocillopora sp.	475	ethanol	
	Amphidinium sp.	475	ethanol	
	*Peridinium cinctum**	475	ethanol	

* Data for *Peridinium cinctum* taken from Strain, Manning, and Hardin (1944).

† Present, but insufficient for spectral analysis.

TABLE IV—*Continued.*

Fraction	Organism	Absorption maxima (nm)	Solvent	Identification
Fraction 8 (brick red)	*Tridacna crocea*	468	ethanol	*Neo-peridinin*
	Hippopus hippopus	465	ethanol	
	Pocillopora sp.	465	ethanol	
	Amphidinium sp.	467	ethanol	
	*Peridinium cinctum**	464	ethanol	
Fraction 9 (pink-orange)	*Tridacna crocea*†			*Unknown*
	Hippopus hippopus†			
	Pocillopora sp.†			
	Amphidinium sp.	460–466	ethanol	
	*Peridinium cinctum**	not present		
Fraction 10 (blue-green)	*Tridacna crocea*	409, 428, 661	ether	*Chlorophyll a*
		410, 428, 662	acetone	
	Hippopus hippopus	409, 428, 663	ether	
	Pocillopora sp.	409, 428, 663	acetone	
	Amphidinium sp.	408, 428, 660	ether	
	*Peridinium cinctum**	not given		
Fraction 11 (light green)	*Tridacna crocea*	448, 582, 634	methanol	*Chlorophyll c*
	Hippopus hippopus	448, 583, 634	methanol	
	Pocillopora sp.	448, 584, 634	methanol	
	Amphidinium sp.	451, 584, 635	methanol	
	*Peridinium cinctum**	not given		

TABLE V

Percentage composition of carotenoids in zooxanthellae from Tridacna crocea, and the dinoflagellate Amphidinium sp.

Fraction number	Pigments	% of Total carotenoids	
		Tridacna crocea	*Amphidinium* sp.
1	β-carotene	3.0	2.5
2	Unknown	1.5	1.7
3 + 4	Diadinoxanthin and dinoxanthin	12.6	10.5
5	Unknown	1.3	0.3
6	Neo-dinoxanthin	3.1	0.6
7 + 8	Peridinin and neo-peridinin	77.0	84.0
9	Unknown	1.5	0.4
		Total 100.0	100.0

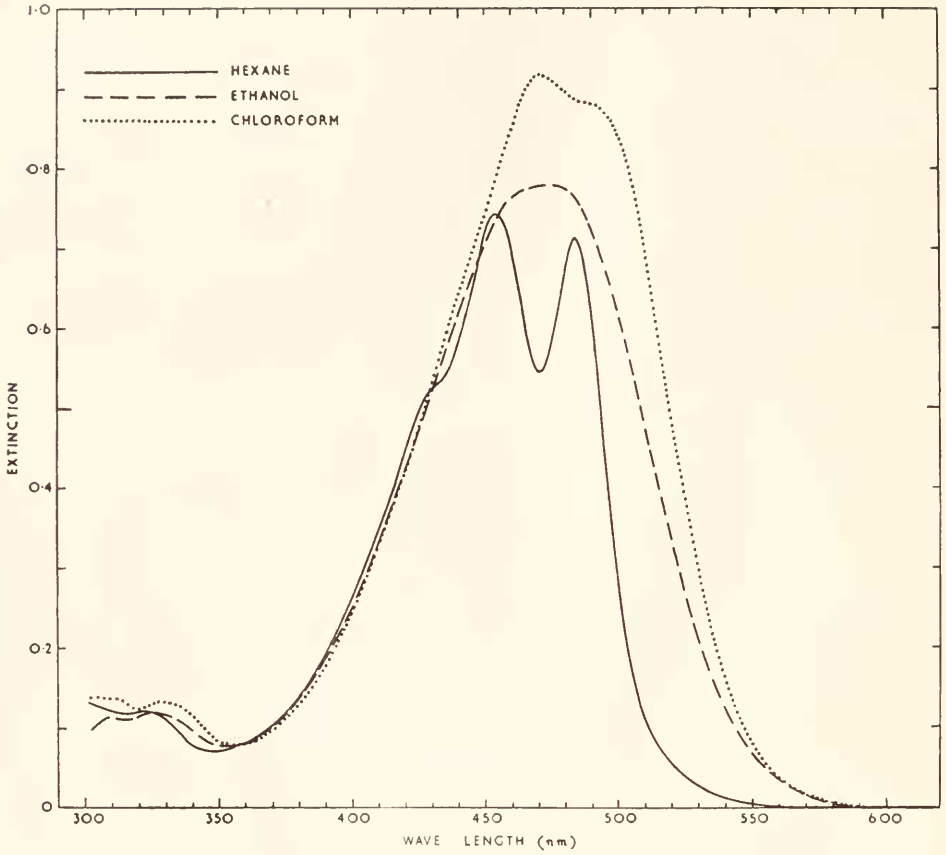

FIGURE 3. Absorption spectra of all-trans peridinin isolated from *Tridacna gigas*.

Peridinin apparently isomerizes fairly rapidly and crystallization of the all-trans fraction may not have excluded traces of the cis-isomers. Cis-trans isomerization of peridinin in benzene solution with iodine caused the maxima to shift from 496 and 468 nm (all-trans) to 493 and 466 nm (equilibrium mixture) after 10 minutes. There was no further spectral change at 120 minutes.

Full absorption curves of peridinin in ethanol, chloroform and hexane are shown in Figure 3. This peridinin was a freshly prepared all-trans fraction from *Tridacna gigas* mantle tissue. Peridinin isolated by paper chromatography from *Pocillipora* zooxanthallae gave similar absorption curves in the three solvents.

The relative proportions of the carotenoids were analyzed by paper chromatography in zooxanthellae from *Tridacna crocea* and in the dinoflagellate *Amphidinum*. Table V shows that in both cases the major xanthophyll fraction, peridinin and neo-peridinin, constituted some 77–84% of the total carotenoids. The other xanthophyll fractions were, by contrast, only a few per cent of the total.

The relative concentrations of chlorophylls *a* and *c* in the zooxanthellae are

given in Table VI. In coral zooxanthellae, the content of chlorophyll *c* was about one-tenth that of chlorophyll *a*. In zooxanthellae from clams, and in the dinoflagellates *Amphidinium* and *Gymnodinium*, the chlorophyll *c* content approached two-thirds that of chlorophyll *a*.

No animal pigments could be detected in the clam mantle tissue itself, identical chromatograms and identical absorption spectra being obtained both with extracts of whole mantle tissue and with isolated zooxanthellae. However, R_f values of the dinoflagellate pigments extracted directly from the mantle were always a little higher than those from zooxanthellae processed separately. That the increased R_f values were due to animal tissue factors extracted with the zooxanthellae was indicated by the effect of adding colorless methanol-acetone extracts of white mantle muscle (without zooxanthellae) to extracts of isolated zooxanthellae cells. R_f values of all pigment fractions became increasingly higher

TABLE VI

Ratio of chlorophyll c:chlorophyll a in zooxanthellae from corals and clams

	Organism	Chlorophyll *c*:*a*
Corals	*Pocillopora* sp.	0.1
	Fungia sp.	0.07
	Acropora sp.	0.2
	Millepora sp.	0.06
Clams		
	Tridacna crocea	0.6
	Tridacna gigas	0.3
	Hippopus hippopus	0.6
Dinoflagellates		
	Amphidinium sp.	0.6
	Gymnodinium sp.*	0.8

* Taken from Jeffrey (1963).

with increasing amounts of animal extract present. This phenomenon, of the presence of colorless factors (probably lipids) influencing the R_f values of photosynthetic pigments, is well documented for other plant pigment systems (Sestak, 1958). Consistent R_f values were, however, always obtained from zooxanthellae which were first separated from the mantle tissue before extraction.

DISCUSSION

Strain, Manning and Hardin (1944) were the first to study in detail the photosynthetic pigments of the free-living dinoflagellates. Using cultures of *Peridinium cinctum*, and separating the pigments on columns of powdered sugar, the components found were chlorophylls *a* and *c*, β-carotene, a major brick-red xanthophyll peridinin and its neo-isomer, and four minor yellow xanthophylls, diadinoxanthin, dinoxanthin, neo-diadinoxanthin, and neo-dinoxanthin. These pigments

were also found in the dinoflagellates *Amphidinium carteri* (Parsons, 1961) *Prorocentrum micans* (Pinckard *et al.*, 1953) and *Gymnodinium* (Jeffrey, 1961) and in zooxanthellae from the sea anemone *Anthopleura xanthogrammica* (Strain, Manning and Hardin, 1944).

Peridinin and dinoxanthin are specific to the dinoflagellates, and have not been found in any other classes of algae. The other pigments (chlorophylls *a* and *c*, β-carotene and diadinoxanthin) are not by themselves taxonomically definitive pigments, since they also occur in other algal groups (Strain, 1958). In the present survey of photosynthetic pigments in zooxanthellae from corals and clams, peridinin was found in every organism tested, and this compound had the same chromatographic and absorption properties as peridinin isolated from the dinoflagellate, *Amphidinium*. In addition, dinoxanthin was found as a minor pigment in all zooxanthellae and dinoflagellate preparations, together with the full complement of chlorophylls and carotenoids originally described by Strain, Manning and Hardin (1944) for *Peridinium cinctum*. The absorption maxima of the pigment fractions from different zooxanthellae corresponded closely to each other and to the published maxima (Table IV). Where small discrepancies were present low extinctions were combined with broad peaks, with consequent difficulties in obtaining accurate maxima. Identification of pigment fractions from their spectral properties was, however, strengthened by the identical R_f values and chromatographic patterns which were obtained.

Peridinin isolated by paper chromatography showed absorption maxima closely similar to freshly prepared all-trans crystalline peridinin. The absorption maxima of peridinin in ethanol from *Tridacna gigas* were at 473 nm (paper chromatography), 473 nm (crystalline "all-trans"), and 472 nm (freshly prepared all-trans), using the Carey spectrophotometer in all cases. With the Beckman DB spectrophotometer, the range of values for peridinin isolated by paper chromatography was 473–475 nm (Table IV). Cis-trans isomerization studies in benzene solution showed that the all-trans fraction had absorption maxima at higher wave-lengths than the individual cis-isomers, or the equilibrium mixture.

The extinction coefficient in acetone of crystalline peridinin from *Tridacna gigas* was substantially higher than that obtained by Parsons and Strickland (1963) for peridinin isolated from *Amphidinium carterei*. The melting point, however, agreed well with the melting point of sulcatoxanthin (peridinin) isolated from the sea anemone, *Anemonia sulcata* (Heilbron *et al.*, 1935). Further, the isomerization behavior of *Tridacna gigas* peridinin resembled similar preparations obtained from *Prorocentrum micans* (Pinckard *et al.*, 1953).

In addition to the major dinoflagellate pigments described, three new xanthophylls were also found in extracts of zooxanthellae and *Amphidinium*. These were readily distinguished both on paper chromatograms and on the sucrose thin-layer plates. Absorption maxima of these fractions taken in ethanol did not appear to correspond to any previously described xanthophyll. Due to the very small quantities present, and the limited duration of the Expedition, it was not possible to analyze these pigment fractions further. Strain, Manning and Hardin (1944) noted a "flavoxanthin-like" xanthophyll in some of their fractions in amounts too small to be analyzed. This may correspond to one or more of the minor xanthophylls described here. Neodiadinoxanthin, found by Strain, Manning,

and Hardin (1944) in *Peridinium cinctum,* could not be located in any of the present preparations studied.

Quantitative data on the carotenoid composition of zooxanthellae and *Amphidinium* showed that the major xanthophyll fraction, peridinin and neoperidinin, constituted some 77–84% of the total carotenoids. Diadinoxanthin was always more prominent than dinoxanthin, and together the concentration was about 10–12%, whereas the other xanthophylls were present in very small quantities which varied from 0.3% to 3.0% of the total. Peridinin in these zooxanthellae (Halldal, 1968; Shibata and Haxo, unpublished data), as well as peridinin from *Gonyaulax polyedra* (Haxo, 1960) appears to have an accessory pigment function in photosynthesis, but the function of the minor carotenoid components, if any, is unknown.

The ratios of chlorophylls *a* and *c* varied depending on whether the zooxanthellae came from a clam or coral host. Clam zooxanthellae resembled the free-swimming dinoflagellates in having a chlorophyll *c* content almost two-thirds that of chlorophyll *a,* whereas in coral zooxanthellae the chlorophyll *c* content was only about one-tenth that of chlorophyll *a.* Further studies would be needed to ascertain whether this was a real difference between clam and coral zooxanthellae. Burkholder and Burkholder (1960) analyzed the chlorophyll content of some alcyonarian corals by spectrophotometric methods and found that chlorophyll *c* was equal to chlorophyll *a.* Using more accurate extinction coefficients the chlorophyll *c* was probably about one-half that of chlorophyll *a.*

No chlorophyll degradation products were ever present on chromatograms of freshly prepared zooxanthellae extracts. This suggests that not only were the extraction and chromatographic procedures reliable, but that the zooxanthellae populations were in a healthy state. Zooxanthellae breakdown must therefore occur at some other locus in the animal host than that sampled, or senescent cells may be discharged from the host tissues and do not accumulate. Traces of pheophytins, chlorophyllides and pheophorbides, indicating the presence of senescent cells, would have been readily detected on the paper chromatograms, had they been present.

Zooxanthellae were further indicated to have dinoflagellate affinities by the presence of gymnodinioid swarmers in suspensions of zooxanthellae which were left overnight in sea water. Although these were not studied in detail, they clearly resembled the swarmers described for *Cassiopea* zooxanthellae, by Freudenthal (1962).

The two-dimensional paper chromatography method provided a simple but useful screening technique for determining the pigment composition of a large range of zooxanthellae. The method was previously used to study the photosynthetic pigments of a variety of different classes of marine algae both in unialgal culture (Jeffrey, 1961; Jeffrey and Allen, 1964), and in natural phytoplankton populations (Jeffrey, 1965). However, for resolution and identification of very closely related carotenoids (*e.g.,* isomers of carotene) thin layer techniques using organic or inorganic adsorbents must be used (Chapman, 1966). In the present work the nature of the carotene fraction was established using thin layers of aluminium and magnesium oxide, and the unknown pink xanthophyll (fraction 9) was separated from chlorophyll *c* on thin layers of polyethylene. The sucrose thin-layer method

had a resolution similar to the separations obtained with the paper method, except that the separation of each component (particularly the xanthophylls) was more complete, and the method may be used for sensitive quantitative analyses.

The present investigation of photosynthetic pigments in clam and coral zooxanthellae establishes that these symbiotic algae contain the full and characteristic complement of dinoflagellate pigments. On the basis of present knowledge of pigment distribution within algal groups, designation of these zooxanthellae as dinoflagellates is clearly justified.

Summary

1. The photosynthetic pigments of the brown symbiotic algae (zooxanthellae) isolated from five tridacnid clams and nine corals were found to be identical with the pigments of the dinoflagellate *Amphidinium*. Identifications were carried out by two-dimensional paper chromatography and by absorption spectrophotometry. Both zooxanthellae and dinoflagellates contained cholorphylls *a* and *c*, β-carotene, peridinin, neo-peridinin, dinoxanthin, neo-dinoxanthin, diadinoxanthin, and three minor xanthophyll fractions not previously described.

2. Peridinin, crystallized from *Tridacna gigas*, or isolated by paper chromatography from *Tridacna crocea*, *Pocillopora* or the dinoflagellate *Amphidinium*, showed similar absorption characteristics in different solvents. The extinction coefficient of crystalline peridinin in acetone was $E_{1\,cm.}^{1\%}$ 1340.

3. Clam zooxanthellae (and the dinoflagellates *Amphidinium* and *Gymnodinium*) contained a much higher proportion of chlorophyll *c* than coral zooxanthellae.

4. No chlorophyll decomposition products were found in any freshly prepared zooxanthellae preparations.

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